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THE ISOLATION AND CHARACTERIZATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA FROM *VIGNA RADIATA L*

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ABSTRACT

Plant and microorganisms relation has a major effect on productivity since human began to rely extensively on agricultural crops for food. Biotechnology has explored the beauty of the microbial world in various fields including agriculture. Soil microbial populations affect plant health and fitness along with soil quality. Plant Growth Promoting Rhizobacteria (PGPR) sets an example in this field. These are underground solutions for above ground problems related to plant. The potentiality of PGPR in agriculture is gradually increasing as it provides an attractive way to replace the use of chemical fertilizers and pesticides. Keeping in view of increasing environmental pollution with a decline in soil health due to use of chemical fertilizers and pesticides, the present study is conducted using bacterial isolates from Vigna radiata for plant growth promotion activities. A total seven strains were isolated and designated as AN12, AN13, AN14, AN15, AN16, AN17 and AN18. Isolated strains were identified with the morphological, biochemical and molecular screening. Antifungal activity was observed by dual culture technique against two phytopathogens viz Fusarium oxysporum and Alterenia solani. Isolated strains were examined for plant growth promoting activities which revealed that some strains are involved in a significant increase in various plant growth parameters. The current study therefore suggested that selected strains of PGPR can be used as biofertilizers as well as biocontrol agents. Hence, these isolates can be further formulated and used for field applications.

KEYWORDS: PGPR, Vigna radiata, Plant Growth Promotion, Biofertilizers, Biocontrol Agents & Phytopathogen

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Original Article

INTRODUCTION

Soil microbial community is the biggest reservoir of biological diversity on this earth so far (Gams 2007). Plant lives in a close association with microbes that inhabit the soil in which plant grows. This area is known as rhizospheric area which is influenced by root secretions and potential source of plant growth promoting rhizobacteria (PGPR). PGPR actively colonize the plant root and enhance the growth and yield of plant by different mechanisms (Schroth and Hancock, 1982). These mechanisms include Phosphate solublization, IAA production, siderophore production, biological nitrogen fixation, HCN production, cytokinins and gibberelic acid production(Glick,1995). Additionally it increases germination percentage, shoot and root growth, total biomass of plant, fodder and fruit yields which can serve as a source of bio fertilizers(Boody and Dobereiner,2000) PGPR strains of Azotobacter, Azospirillum, Bacillus, Burkholderia, Klebsiella, Pseudomonas, Rhizobium, Bradyrhizobium and serratia have been reported to increase plant growth, yield and nutrient content in various crop plants(Joseph et al 2007;Mia et al.2010). PGPR are nonpathogenic but possess various mechanisms to suppress the plant pathogens like competing for a fundamental niche (Elad et al. 1985), antibiosis by producing antibiotics and hydrogen cyanide (HCN) and also acting as a good source of siderophores which chelate the iron in vicinity of root

to limit the iron availability which is necessary for phytopathogens growth (Kleopper et al. 1980).

Consumption of legumes constitutes about 5% of all cultivated crops for human. Leguminous crops are rich in fibres, calcium, phosphorus, iron, vitamins and many essential amino acids. *Vigna radiata* (mung bean) is one of the nutritious and economically important leguminous crops in India. Knowledge of the native microbial population, their characterization and identification is necessary for understanding the distribution and diversity of microbes in the rhizosphere of specific crops (Chahboune et al. 2011). So, in present study effects of isolated PGPR on *Vigna radiata* has been evaluated.

METHODOLOGY

Collection of the Samples

A collection of rhizospheric soil samples had been done from the fields of *Vigna radiata L.* grown and cultivaed in Meyar village of Nalanda district in Bihar, India. Rhizospheric soils tightly adhering to roots of *Vigna radiata* had been collected during vegetative and flowering stages showing good and healthy growth. Samples were stored in sterilized sample collection bags and packed for transport to the laboratory for further study.

Isolation of Bacteria

The rhizospheric soil samples had been processed within 24 hours for isolating most predominant PGPR by serial dilution technique. Fresh roots were washed under running tap water and then surface sterilized with 5% NaOcl. After surface sterilization the root samples were again washed three times with sterile distilled water. The root samples were crushed with mortar and pestle. Serial dilutions were prepared from grounded roots.

Soil tightly adhering to the roots of *Vigna radiata* were serially diluted for isolating microorganism from rhizosphere. 0.1ml of the alliquotes were transferred to different culture media which were Nutrient agar, Luria-Bertini (LB) agar, SCA, Macconky agar and spreaded over the entire media surface.

Plates were then incubated at 37^{oC} till the appearance of different colonies. Morphologically different colonies were picked and streaked on fresh medium in order to obtain a pure culture.

Morphological and Biochemical Characterization

Seven bacterial colonies were isolated and coded as AN12, AN13, AN14, AN15, AN16, AN17, AN18, and subjected to microscopic examination. Colony morphology (size, shape, colour, margin, elevation and texture) were observed with the oil immersion objective of the bright field microscope. The Grams reaction was performed as per standard protocol. Biochemical characterizations of all the isolates such as indole, Methyl red, Voges-Proskauer test, citrate, oxidase and catalase were examined according to standard methods (Cappuccino et al., 1992). The microbial isolates were identified in accordance with the Bergey' s manual of determinative bacteriology (Holt et al., 1994).

Molecular Characterization

Morphological and biochemical characterization are sometimes not much helpful for identification of all isolates. Strains which were not identified by above were subjected to identification based on 16S rRNA sequencing. DNA was isolated from the culture by the CTAB method and quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed. Isolated DNA was amplified with *16S rRNA* Specific Primer (**8F**and **1492R**) using Veriti® 99 well Thermal Cycler (Model No. 9902). A single discrete PCR amplicon band was observed. The PCR

amplicon was enzymatically purified and further subjected to Sanger Sequencing. The bi-directional DNA sequencing reaction of PCR amplicon was carried out with **8F**and **1492R** primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of *16S rDNA* was generated from forward and reverse sequence data using aligner software. The *16S rDNA* sequence was used to carry out BLAST alignment search tool of NCBI Genbank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou et al., 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The percentage of replicating trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

Screening of Antifungal Activity

Strains were subjected to dual culture technique(Berg et al 2002) using PDA media(Dennis and Webster,1971) for their evaluation of efficacy against two fungal phytopathogen *Fusarium oxysporum* and *Altereniasolani*.5 mm mycelial disc of seven day old culture was placed on to the center of the Petri dish and the isolated PGPR strains were spread 2 cm away on either side of mycelial disc. Petri dishes inoculated with fungal discs alone served as control. The plates were incubated at 28^0C for about 5 – 7 days or until the leading edge of fungus in the control plate reached the edge of the plate. Three replications were maintained for each isolate. The radial growth of fungal mycelium was measured and percent inhibition of growth over untreated control was estimated. The percent inhibition was compared with control using the formula proposed by Vincent (1927) Percent inhibition (I) = C-T/C

Where,

C = mycelial growth of pathogen in control

T = mycelial growth of pathogen in dual culture plate.

Green House Evaluation of PGPR Strains for Growth Promotion Activities

For the study plastic pots of size 13X12X5 inches were filled with sieved clay loamy soil(< 2 mm) having pH6.6. Treatments included isolated PGPR alone as well as in combination of two or more PGPR strains. Two combinations named consortium (CONS.1) 1 and consortium 2 (CONS.2) were made. Consortium 1 included AN13, AN16 and AN17 whereas consortium 2 included the combination of AN 15 and AN17. The study also includes an absolute control with only soil that is without PGPR. The experiment was replicated 5 times. Seeds of *Vigna radiata* were treated with a broad range of fungicide carbendazim at 2 g kg^{-1} before treatment with PGPR suspension. Seeds were soaked in 1% starch solution containing a respective PGPR suspension(~$X10^7$cfu mL^{-1}) for 1h shade dried for 24 h and sowed @ one seed per pot. All the pots were applied with major nutrients at rate equivalent to the recommended dose (RD) of NPK for *Vigna radiata*

which is 5-10-15 kg $ha^{-1.}$ Booster dose of each PGPR was given at 30 and 60 days after planting (DAP) @ 0.5 L per pot (10^8cfu mL^{-1}). The inorganic sources of NPK used were urea, rock phosphate(RP) and muriate of potash(MOP) respectively. Five replications were maintained for each treatment.

Growth promoting observations like height of plant, root length were recorded. Root length was measured from the attachment point of stem base to the tip of the adventitious root. The statistical analysis was done using OPSTAT(Sheoran, 1998) and expressed as the mean of five independent replications ± standard error (SE) along with analysis of variance (one way ANOVA) at the $p \leq 0.05$ significance level.

RESULTS AND DISCUSSIONS

Isolation of different rhizobacterial strains from *Vigna radiata L.* were carried out by standard techniques. Total seven rhizobacterial isolates were isolated and differentiated by their morphological and biochemical characters. The result shows different colony morphologies. Colony size ranges from pinpoint, small and large. Whereas shape includes round, circular and irregular. Margin is entire and wavy. Elevation of isolates includes convex, raised and umbonate. Texture is generally found mucoid and dry. Morphological characters have been presented in Table1. Biochemical characters of isolates varied among isolates. Out of seven, two strains shows Gram positive, while four strains shows Gram negative reaction and one strain is Gram variable. One strain is indole positive and six strains are indole negative. Methyl red test revealed that one isolate is positive and six are negative. Three isolates were VP positive and four are negative. Simmon's citrate shows that all seven isolates are citrate positive. Four strains were oxidase positive whereas three were negative. All seven strains are catalase positive. The biochemical characteristics of rhizobacterial strains have been presented in Table 2.

Table 1: Colony Morphology of Isolates from Rhizosphere of *Vigna Radiata L*

S. No.	SIZE	Colony Shape	Margin	Elevation	Texture	Gram Staining
A.N.12	Large	Irregular	Entire	Convex	Mucoid	__
A.N.13	Small	Circular	Entire	Convex	Mucoid	__
A.N.14	Small	Round	Entire	Convex	Mucoid	__
A.N.15	Large	Circular	Wavy	Umbonate	Mucoid	Variable
A.N.16	Large	Circular	Entire	Raised	Mucoid	+
A.N.17	pinpoint	Circular	Wavy	Convex	Dry	__
A.N.18	Large	Irregular	Wavy	Umbonate	Dry	+

Table 2: Biochemical Assay of Isolates from Rhizosphere of Vigna Radiata L

S. No.	Indole	Methyl Red	Voges-Proskauer	Citrate	Oxidase	Catalase
A.N. 12	__	__	__	+	+	+
A.N. 13	__	__	__	+	__	+
A.N. 14	__	+	__	+	__	+
A.N. 15	__	__	+	+	+	+
A.N. 16	+	__	__	+	+	+
A.N. 17	__	__	+	+	__	+
A.N. 18	__	__	+	+	+	+

The identification of the isolates was done using the morphological and biochemical properties with the help of Bergey's Manual of Determinative Bacteriology. AN12 was identified as *Achromobacter xylosoxidans*, AN13 as *Bradyrhizobium japonicum*, AN14 as Proteus mirabilis, AN16 as *Azootobacter chroococcum*, AN17 as *Burkholderia cepacia* and AN 18 as *Bacillus subtilis.* Rhizobacterial strains AN15 was identified by 16S rRNA gene sequence. Isolated DNA was amplified and enzymatically purified and quality checked. A single discrete band of 1500 bp was found as

shown in figure 1. Purified PCR amplicon were further processed or Sanger sequencing and consenus sequence of 1299 bp in case of AN4,1488 bp in case of AN5 and 1301 bp 16S rDNA was generated from forward and reverse sequence data using aligner software.

Based on the sequences of strain AN15, BLAST search result found thatAN15 showed similarity with *Lysinibacillus macroides* strain Xi9 (Accession number: KY317957.1). (Figure 1)Accession number for nucleotide sequence of AN15 is MG493188.

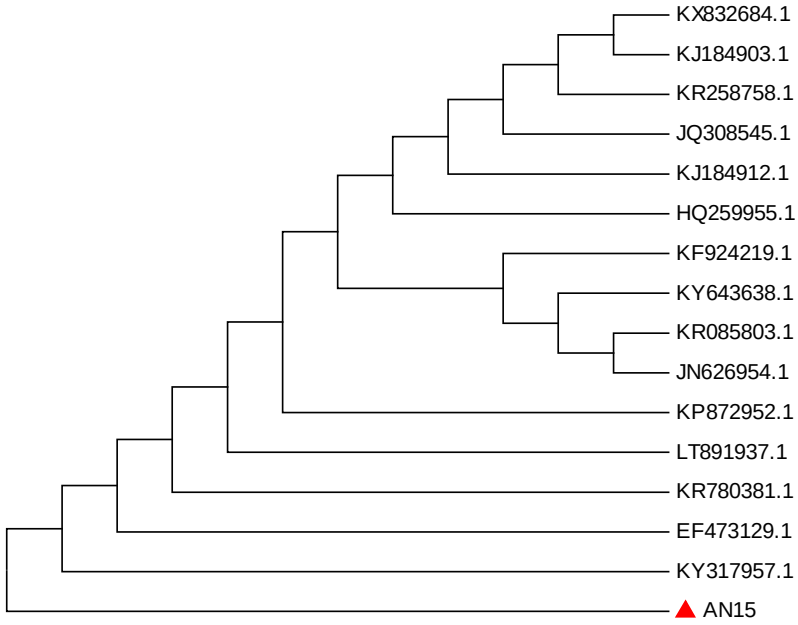

Figure 1: Phylogenetic Tree of Strain AN15

In vitro screening of isolated strains clearly indicated the potential of strains AN12, AN15, AN17 and AN18 to inhibit the phytopathogen *Fusarium oxysporum*, while *Alterenia solani* was inhibited by AN 12 and AN15. AN15 showed maximum growth inhibition percentage against both *Fusarium oxysporum*(58.53%)and *Alterenia solani*(51.51%) (Tables 3 and 4).

Table 3: Antagonistic Activity of Rhizobacterial Strains against *Fusarium oxysporum and*

Rhizobacterial Isolates	Mycelial Growth (mm)	Growth Inhibition (%)
AN12	40	51.21
AN15	34	58.53
AN 17	57	30.48
AN18	39	52.43

Table 4: **Antagonistic Activity of Rhizobacterial Strains against *Altereneria solani***

Rhizobacterial Isolates	Mycelial Growth (mm)	Growth Inhibition (%)
AN12	40	39.39
AN15	32	51.51

PLANT GROWTH PARAMETERS

The bacterial isolates exerted a significant influence on *Vigna radiata* growth characteristics especially when applied in a combination form. Comparisons were made among isolated strains, non-inoculated control and in combination of strains. The ANOVA analyses had been presented in table 5.

Table 5: Crop Growth Parameters Studies in Pots under Glass House Conditions

Isolates	Shoot Length(cm)	Root Length(cm)
AN12	47.28 ± 0.32	23.02± 0.57
AN13	47.86 ± 0.38	24.64± 0.66
AN14	36.04 ± 0.73	16± 0.67
AN15	47.08 ± 0.24	22.92 ± 0.81
AN16	46.14 ± 0.38	22.04 ± 0.65
AN17	48.98 ± 0.47	24.02 ± 0.79
AN18	45.98 ± 0.45	22.38 ± 0.61
CONTROL	44.52 ± 0.47	23.34 ± 0.59
CONS. 1	57.48 ± 0.48	26.74 ± 0.44
CONS. 2	54.44± 0.71	24.38 ± 0.61

CONCLUSIONS

The present study underlines the importance of isolating and screening PGPR for multiple plant growth promotion and biocontrol traits. The study evaluates promising strains through greenhouse pot experiments in *Vigna radiata*. However, it is evident from the study that the ability of a strain to promote the growth varied among different strains and was much more effective when performed in a specific combination. This may be because of variation in the dynamics of rhizosphere of a given bacterial isolate, which in turn may be due to the change in exudates pattern in the root region of the plant. The combination of isolates found to be viable, efficient and effective alternatives for improved growth and nutrient uptake. The data generated provide useful information for developing appropriate biofertilizer technology.

CONFLICT OF INTEREST

There is no any conflict of Interest of the corresponding author and all authors for Publication of Article.

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